

Symbiotic nitrogen fixation in black locust (*Robinia pseudoacacia* L.) seedlings from four seed sources

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Abstract: We conducted a greenhouse experiment to investigate the role of seed source in growth and symbiotic nitrogen fixation of black locust (*Robinia pseudoacacia* L.). Seeds from different sources were planted in the same environmental conditions and inoculated with a suspension of mixed *Rhizobium*. We used the modified ^{15}N isotope dilution method to estimate biological nitrogen fixation of *Robinia* trees. Different *Robinia* seed sources differed significantly in terms of tissue dry weight (50.6–80.1 g), total N (1.31–2.16 g) and proportion of nitrogen derived from the atmosphere (0–51%). A higher nitrogen fixation rate of *Robinia* trees was associated with higher dry weight. Moreover, the leaves of *Robinia* proved to adequately represent the nitrogen fixation capacity of entire plants. Our results confirmed that assessment of seed sources is a useful way to improve the nitrogen fixation capacity and therefore the growth rate of *Robinia*.

Keywords: *Robinia pseudoacacia*; seed source; ^{15}N dilution method; symbiotic nitrogen fixation

Introduction

Black locust (*Robinia pseudoacacia* L.) is native to North America (Barrette et al. 1990) and is one of the most effective nitrogen-fixing tree species in the world (Olesniewicz et al. 1999). It is economically and ecologically important, and has been cultivated widely in the world with a global area of plantations of three million hectares (Hanover et al. 1991). The uptake of N from soils declines when nitrogen fixing plants fix atmospheric

N (Giller et al. 1991) and this enhances soil fertility, particularly in poor soils of arid regions.

A number of factors affecting nitrogen fixation have been investigated in *Robinia*, including seasonal variation (Boring et al. 1984), CO_2 concentration (Feng et al. 2004), symbiotic microbes (Tian et al. 2003), and soil properties such as nutrient availability, soil moisture, soil temperature, and pH (Noh et al. 2010; Berthold 2005). But few studies have investigated the effect of *Robinia* seed sources in nitrogen fixation.

Provenance is defined as the place where trees of one species grow (whether native or not) (Ford Robertson 1971). Provenance of seed has been shown to affect nitrogen fixation ability, and has been described for *Acacia nilotica* and *Faidherbia albida* (Beniwal et al. 1995; Gueye et al. 1997). The objective of this study was to investigate whether provenance of seed affects nitrogen-fixing capacity of *Robinia*.

Material and methods

Collecting seeds and greenhouse experiment

We selected four *Robinia* seed sources in Iran and Hungary (Table 1). At each seed collecting area, 15 trees were randomly selected from even-aged single-family stands of *Robinia*. Seeds were collected from the top, middle, and bottom of the canopy and then mixed together to yield a composite sample.

We use the collected seeds to conduct greenhouse trials between November 2008 and May 2009 in Goettingen, Germany, located at 51°33' N and 9°57' E and 205 m elevation. Seeds were germinated after scarifying using abrasive paper and soaking in water overnight. After three weeks, seedlings were transplanted into pots containing commercial potting soil (pH: 5.9, KCl: 2 g·L⁻¹, organic material: 35 %, nitrogen: 300 mg·L⁻¹, P₂O₅: 240 mg·L⁻¹, K₂O: 350 mg·L⁻¹). Then the seedlings were inoculated with a suspension of mixed *Rhizobium* from commercial inocula at a level of approximately one million bacteria of each strain per plant (strains Rob 8, Rob 10, Rob 11, Rob 12, Rob 16 and Rob 25; Johann Heinrich von Thünen-Institut in Waldsiedersdorf, Germany). The greenhouse temperature was maintained at 20°C

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and the photoperiod was eight hours per day. All the plants received equal amounts of water two times per week.

A Completely Randomized Design (CRD) was used, consist-

ing of four treatments of *Robinia* seed sources, one for each collection provenance (i.e. Hosszúpályi, Karaj, Sanandaj and Semnan). We replicated each treatment six times.

Table 1. Locations of the *Robinia* seed collecting provenances

Origin of seeds (Country/City)	Age of planta- tion (years)	Elevation(m)	Latitude	Longitude	Mean annual temperature (°C)	Mean annual precipitation (mm)	Period of sun- shine (h·a ⁻¹)	
Iran	Semnan	31	1117	35°36' N	53°30' E	18.3	141	3018
	Karaj	26	1275	35°44' N	51°10' E	15.8	244	2952
	Sanandaj	31	1397	35°14' N	47°00' E	14.2	459	2829
Hungary	Hosszúpályi	35	105	47°24' N	21°45' E	10.1	567	1982

Sampling and chemical analysis

At six months from the start of the greenhouse trials, seedlings were harvested and separated into leaves, stems, and roots. Samples were washed with distilled water, dried at 60°C for 48 h, weighed, and ground. Atom % ¹⁵N excess and nitrogen (N%) measurements were conducted at the Center for Stable Isotopes Research and Analysis (KOSI) of Goettingen University using an isotope ratio mass spectrometer (Delta C, Finnigan MAT GmbH, Bremen, Germany).

Estimation of Symbiotic nitrogen fixation

We used the modified ¹⁵N isotope dilution method to compare biological nitrogen fixation between *Robinia* seed provenances (Danso et al. 1993). The calculation of nitrogen fixation was based on the following equation:

$$N_{dfa} = (1 - (Fp_{Nae} / Rp_{Nae})) \times 100 \quad (\text{Fried et al. 1977}) \quad (1)$$

where: N_{dfa} is the percentage of nitrogen derived from the atmosphere (%); Fp_{Nae} is Atom % ¹⁵N excess in fixing plant organs; Rp_{Nae} is Atom % ¹⁵N excess in reference plant organs.

The highest ¹⁵N enrichment of the four provenances proved to be in tissues from Semnan. This value was used as a reference to estimate the relative least possible quantitative N₂ fixed by plants (Danso et al. 1993). Thus the values of %Ndfa and Ndfa related to Semnan are shown as 0.00.

Statistical analyses

The data were analyzed using one-way ANOVA, followed by comparisons of means using the Duncan test at 95% confidence. STATISTICA software version 8.0 (StatSoft 2008) was used for statistical analysis.

Results

Average tissue dry weight varied significantly between the four provenances of *Robinia*, ranging from 51 g to 83 g (Table 2, Fig. 1). Plants grown from seeds from Hosszúpályi had significantly

greater dry weight for all plant parts (i.e. leaves, stems and roots). Differences between other provenances were mainly seen in stem dry weight. On average, the total dry weight of *Robinia* was distributed as 25.1%, 38.3% and 36.6% in roots, stems and leaves, respectively.

Table 2. One-way ANOVA of seeds source effects on dry weight, total N, % Ndfa, and Ndfa of whole trees of *Robinia*

Variable	SS	d.f.	MS	F-value	P-value
Dry weight	3083.661	3	1027.882	30.923	0.000
Error	664.762	20	33.238		
Total N	2.434	3	0.811	162.922	0.000
Error	0.099	20	0.004		
Ndfa	0.400	2	0.200	96.455	0.000
Error	0.031	15	0.002		
% Ndfa	359.650	2	179.825	9.264	0.002
Error	291.146	15	19.409		

Notes: Ndfa is nitrogen derived from atmosphere.

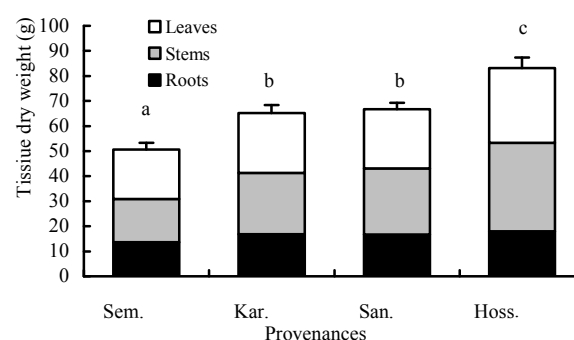


Fig. 1 Mean dry weights of *Robinia* tissues by seed provenance and plant part

Total nitrogen content of *Robinia* plants from different seed sources varied significantly, 1.31–2.16 g·plant⁻¹ (Table 2, 3). Leaves accumulated the highest percentage of total N (mean for all seed sources = 51%), while stems showed the greatest variation in total N between seed sources (Table 3).

The highest level of variation in the %¹⁵N between *Robinia* seed sources was detected in leaves. Semnan plants had the highest leaf %¹⁵N content (Table 3). In contrast, atom %¹⁵N content

did not differ by seed provenance. The proportion of N derived from nitrogen fixation (%Ndfa) varied significantly by seed provenance (0–51%, Table 2, 3). Leaves showed the highest degree of variation in %Ndfa between seed sources, while no difference was observed between roots (Table 3). The amount of N derived from nitrogen fixation (Ndfa) also differed significantly by seed provenance (0–0.96 g·plant⁻¹, Table 2, 3).

Table 3. Mean (±SD) total nitrogen, atom%¹⁵N percentage (%Ndfa), and amount (Ndfa) of symbiotic nitrogen fixed in different tissues of *Robinia* seed sources

Plant parts	Seed sources	Total N (g·plant ⁻¹)	Atom% ¹⁵ N	% Ndfa	Ndfa (g·plant ⁻¹)
Leaf	Semnan	0.81a (0.07)	0.94c (0.04)	0.00 (0.00)	0.00 (0.00)
	Sanandaj	0.75a (0.02)	0.65b (0.02)	34.68a (3.13)	0.26a (0.02)
	Karaj	0.72a (0.04)	0.46a (0.12)	53.66c (5.28)	0.40b (0.02)
	Hosszúpályi	1.03b (0.08)	0.59ab (0.12)	42.95b (3.59)	0.44b (0.03)
Stem	Semnan	0.20a (0.02)	1.06b (0.17)	0.00 (0.00)	0.00 (0.00)
	Sanandaj	0.40b (0.04)	0.67a (0.06)	38.76a (3.67)	0.16a (0.01)
	Karaj	0.32b (0.02)	0.56a (0.14)	49.80b (3.07)	0.16a (0.10)
	Hosszúpályi	0.55c (0.08)	0.57a (0.06)	46.48b (4.56)	0.26b (0.03)
Root	Semnan	0.29a (0.02)	1.23b (0.19)	0.00 (0.00)	0.00 (0.00)
	Sanandaj	0.36a (0.03)	0.63a (0.04)	48.37a (3.37)	0.17a (0.01)
	Karaj	0.50b (0.08)	0.63a (0.11)	48.27a (5.88)	0.24b (0.01)
	Hosszúpályi	0.58b (0.07)	0.68a (0.12)	44.34a (3.81)	0.26b (0.05)
Whole plant	Semnan	1.31a (0.05)	1.11b (0.15)	0.00 (0.00)	0.00 (0.00)
	Sanandaj	1.51b (0.07)	0.64a (0.03)	39.89a (3.16)	0.60a (0.04)
	Karaj	1.54b (0.06)	0.55a (0.12)	50.81c (4.17)	0.78b (0.04)
	Hosszúpályi	2.16c (0.10)	0.61a (0.06)	44.59b (4.20)	0.96c (0.05)

Notes: Different letters in same columns indicate significant ($p \leq 0.05$) differences among seed sources; Ndfa is nitrogen derived from atmosphere.

Discussion

Seed provenance strongly affected growth and nitrogen fixation of *Robinia* seedlings. Dry weight of seedling tissues increased

with increasing nitrogen fixation rate (Table 3 and Fig. 1). This study also demonstrated high rates of fixation of atmospheric nitrogen in *Robinia* (over 45% of total nitrogen fixation) within six months of planting. The leaves of *Robinia* seedlings proved to be useful indicators of the proportion of nitrogen fixation, as the leaves yielded %Ndfa values similar to those documented for entire seedlings (Table 3).

Variation in nitrogen fixation between seed provenances of tree species has been shown for *Casuarina equisetifolia* and *Casuarina cunninghamiana* (Sanginga et al. 1990a), *Acacia albida* (Sanginga et al. 1990b), and *Acacia senegal* (Raddad et al. 2005). Positive correlation between nitrogen fixation and dry weight of plants was also shown by Sanginga et al. (1990b). Our results confirmed those of Gueye et al. (1997) who observed that some seed sources of *Faidherbia albida* produced plants of similar dry weight, but different nitrogen fixing capacity. Our results also confirmed those of earlier studies showing that leaves can be indicative of nitrogen fixation by the whole plant (Danso et al. 1995; Sylla et al. 1998; Raddad et al. 2005). Some studies, however, demonstrated that proportions of fixed nitrogen differed by plant part (Butler 1987; Ladd 1981).

The results of this study can be used to improve the capacity for nitrogen fixation of *Robinia* by assessing available local or introduced seed sources for their nitrogen fixing capacity. The selection of superior seed sources provides a great benefit in arid regions, where soil fertilization or conservation is the major issue, and nitrogen is the primary limiting resource for plant growth (Krueger-Mangold et al. 2004; Paschke et al. 2000). The correlation between nitrogen fixing capacity and biomass of *Robinia* can be applied as an indirect method to differentiate *Robinia* genotypes, seed sources, or other treatments. Biomass of *Robinia* plants can be a good indicator of their nitrogen fixing capacity when the use of ¹⁵N-labeling methods is not possible due to high costs or lack of facilities.

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